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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 32, Issue of August 12, pp. 20662–20667, 1994
Printed in U.S.A.

Functional Properties of Human Factor Va Lacking the Asp^{683}–Arg^{709} Domain of the Heavy Chain*

(Received for publication, December 21, 1993, and in revised form, May 4, 1994)

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A protease purified from the venom of the elapid snake *Naja naja oxiana* converts human blood coagulation factor Va into a molecule (factor Va_{NO}) with greatly reduced cofactor activity. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed that the venom protease cleaved a small peptide from the heavy chain of factor Va and reduced the apparent M_r from 105,000 to 101,000. This peptide was isolated by high performance liquid chromatography on a reversed-phase column. Amino acid sequence analysis of the peptide indicated that the venom enzyme cleaved the peptide bond between His^{682} and Asp^{683}, thus removing 27 amino acids from the carboxyl-terminal part of the heavy chain. The cofactor activities of factors Va and Va_{NO} were compared by measuring their abilities to support factor Xa-catalyzed prothrombin activation in the presence of phospholipids and calcium ions. Both factor Va molecules stimulated the binding of factor Xa to negatively charged phospholipids. However, the amounts of factor Va required for half-maximal incorporation of factor Xa into the membrane-bound factor Xa-Va complex were much lower for native factor Va (0.25 nM) than for factor Va_{NO} (2.01 nM). At saturating concentrations of factor Va or factor Va_{NO} the k_{cat} values for prothrombin activation were 114 s^{-1} for factor Va and 128 s^{-1} for factor Va_{NO}. The K_m values for prothrombin determined under these conditions were 0.24 and 0.83 μM for prothrombinase complexes with native factor Va and factor Va_{NO}, respectively. Direct binding studies revealed that factors Va and Va_{NO} bind with equal affinity to phospholipids. These data indicate that factor Va_{NO} is impaired in its ability to interact with factor Xa and prothrombin. Together with the structural data this implies that the carboxyl-terminal Asp^{683}–Arg^{709} domain of the heavy chain is required for optimal interaction of factor Va with factor Xa and prothrombin.

Blood coagulation factor V is a single-chain glycoprotein with a molecular weight of 330,000 (1, 2) that contains three different types of protein domains (A, B, and C) arranged in the order A1-A2-B-A3-C1-C2 (3, 4). Activation of human factor V by thrombin results in the removal of the major part of the B domain and the generation of factor Va, a molecule that consists of a heavy chain (containing the A1, A2, and a small piece of the B domain) and a light chain (containing the A3, C1, and C2 domains) that are associated via a single Ca^{2+} ion (5, 6).

Factor Va is a nonenzymatic protein cofactor, which together with the serine protease factor Xa, Ca^{2+} ions, and a procoagulant membrane surface forms the prothrombinase complex. Depending on the reaction conditions the presence of factor Va causes a 10^3-10^6-fold stimulation of the rate of prothrombin activation (7, 8). This rate enhancement appears to be caused by 1) an increase of the catalytic activity (k_{cat}) of the enzyme factor Xa (7, 8), 2) stimulation of the binding of factor Xa to phospholipid membranes (7, 9, 10), and 3) an increased interaction of the substrate prothrombin with the prothrombinase complex (10).

Snake venom proteins have been shown to be helpful tools in studying structure-function relationships of blood coagulation factors. Recently, we have purified a protease from the venom of *Naja naja oxiana* (11) that is able to activate both human and bovine factor V. However, the factor Va molecule generated by this venom activator exhibited a much lower cofactor activity than thrombin-activated factor V. Actually, the venom protease can also be regarded as a factor Va inactivator because incubation of thrombin-activated human factor Va with the venom protease resulted in an 80–90% loss of cofactor activity.

In the present paper we describe the structural and functional properties of the factor Va derivative obtained after incubation of factor Va with the venom protease. It is shown that the protease cleaves a 27-amino acid peptide (Asp^{683}–Arg^{709}) from the carboxyl-terminal part of the heavy chain of factor Va. The corresponding loss of cofactor activity appears to be caused by impaired interaction of venom-treated factor Va with prothrombin and factor Xa.

EXPERIMENTAL PROCEDURES

Materials—Hepes, Tris, bovine serum albumin, chicken egg albumin (ovalbumin), soybean trypsin inhibitor (type IS), bovine brain phosphatidylserine, egg yolk phosphatidylcholine, and Russell's viper venom were purchased from Sigma. DOPC[1] and DOPS were obtained from Avanti Polar Lipids, Pelham, AL. The chromogenic substrates S2238 and S2337 and the thrombin inhibitor I2581 were supplied by Chromogenix, Mölndal, Sweden. D-Pro-Phe-Arg-CH_2Cl was obtained from

* This work was supported in part by Program Grant 900-526-192 from the Dutch Organization for Scientific Research (NWO). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a grant from the Committee for Cooperation with Eastern Europe from the Dutch Organization for Scientific Research (NWO).

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[1] The abbreviations used are: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; factor Va_{NO}, factor Va treated with the protease purified from *N. naja oxiana* venom; BSA, bovine serum albumin; I2581, *N*-dansyl-(*p*-guanidino)-phenylalanine-piperidide hydrochloride; S2238, D-Phe-(pipecolyl)-Arg-*p*-nitroanilide; S2337, Ile-Glu-(piperidyl)-Gly-Arg-*p*-nitroanilide; *p*-NPGB, *p*-nitrophenyl-*p*′-guanidinobenzoate hydrochloride; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; RVV-X, purified factor X activator from Russell's viper venom.

Calbiochem, and *p*-NPGB was from Nutritional Biochemicals. Fast protein liquid chromatography equipment and column materials used for protein purification were purchased from Pharmacia, Uppsala, Sweden.

Proteins—Human coagulation factors used in this study were isolated from fresh frozen plasma. Human prothrombin and factor X were purified according to DiScipio *et al.* (12). Human thrombin was prepared from prothrombin activation mixtures as described by Pletcher and Nelsestuen (13). Human factor Xa was obtained from purified factor X after activation with RVV-X and isolation from the activation mixture by affinity chromatography on soybean trypsin inhibitor-Sepharose (14). RVV-X was purified from Russell's viper venom according to Schiffman *et al.* (15). Human factor V was purified essentially as described by Dahlbäck (1) with minor modifications (16). Factor Va was obtained after activation of factor V with thrombin, separated from activation peptides on a prothrombin-CL4B-Sepharose column (17), and stored in a buffer containing 25 mM Hepes pH 7.5, 100 mM NH_4Cl, 5 mM $CaCl_2$, and 5 mg/ml BSA. The purified coagulation factors were stored at −80 °C. Protein preparations were >95% pure as judged by SDS-polyacrylamide gel electrophoresis according to Laemmli (18).

Protein Concentrations—Protein concentrations were determined according to Lowry *et al.* (19). Molar thrombin and factor Xa concentrations were determined by active-site titration with *p*-NPGB (20, 21). Prothrombin concentrations were determined after complete activation of prothrombin with *Echis carinatus* venom and quantitation of thrombin with *p*-NPGB. Factor V concentrations were estimated from the absorbance at 280 nm using an $A_{280}^{1\%}$ of 8.9 (2). Factor Va was quantitated as described below.

High Performance Liquid Chromatography—Reversed-phase chromatography was performed on an Aquapore C18 (2.1-mm internal diameter) column connected to a microbore HPLC system from Applied Biosystems at a flow rate of 200 µl/min. After application of the samples, the proteins and peptides were eluted with a linear gradient of solvent B (0.1% trifluoroacetic acid in 60% acetonitrile) in solvent A (0.1% trifluoroacetic acid). Proteins and peptides present in the eluate were detected by measuring the absorbance at 214 nm. Further details are given in the legend to Fig. 2.

Amino-terminal Sequence Analysis—200-µl samples containing either (*a*) 6 µg of factor Va or (*b*) 6 µg of factor Va treated with 0.06 µg of venom protease or (*c*) 0.06 µg of venom protease were adsorbed onto Prospin polyvinylidene difluoride filters (Applied Biosystems), and the filters were washed by centrifugation with methanol/H_2O (20:80). In the case of amino acid sequence analysis of peptide fractions obtained after HPLC (*cf.* Fig. 2) the peptide-containing fraction was freeze-dried, redissolved in 0.1% trifluoroacetic acid, and loaded on Polybrene-coated glass filters.

The samples were subsequently subjected to automated amino-terminal sequencing on an Applied Biosystems model 476A pulsed liquid sequencer.

Phospholipid Vesicle Preparations—Appropriate quantities of phospholipids dissolved in chloroform/methanol (1:1, v/v) were mixed in a small glass tube. After drying under a mild flow of N_2 the phospholipids were suspended in 2 ml of a buffer containing 25 mM Hepes pH 7.5 and 175 mM NaCl. The phospholipid suspension was vigorously vortexed for 1 min and subsequently sonicated for 10 min at 0 °C using an MSE Mark II 150-watt ultrasonic disintegrator set at 8 µm peak to peak amplitude. Phospholipid concentrations were determined by phosphate analysis (22).

Factor Va Assay—Factor Va was quantitated by determining the rate of factor Xa-catalyzed prothrombin activation in reaction mixtures that contained a limiting amount of factor Va and saturating concentrations of phospholipid vesicles (50 µM phosphatidylserine/phosphatidylcholine, 10:90, M/M), factor Xa (5 nM), and prothrombin (0.5 µM) (16). The molar factor Va concentration in the assay mixture was calculated from the rate of prothrombin activation using a turnover number of 6000 mol of prothrombin activated per min per mol of factor Xa-Va complex (23). Molar venom-treated factor Va (Va_{NO}) concentrations were calculated from and taken to be identical to the factor Va concentration initially present.

The assay conditions were modified in experiments in which differences in cofactor activity between factors Va and Va_{NO} were monitored. For optimal display of functional differences between factors Va and Va_{NO}, the factor Va assay was performed at suboptimal factor Xa (0.5 nM), phospholipid (5 µM phosphatidylserine/phosphatidylcholine, 5:95, M/M), and prothrombin (0.25 µM) concentrations (see legend to Fig. 1).

Kinetic Data Analysis—The formation of a membrane-bound factor Xa-Va complex was determined by measuring rates of prothrombin activation in the presence of phospholipid vesicles at a fixed (limiting) concentration of factor Xa and varying amounts of factor Va or vice versa. Factor Xa, factor Va, and phospholipid vesicles were preincubated for 5 min at 37 °C in 25 mM Hepes pH 7.5, 175 mM NaCl, 2 mM $CaCl_2$, and 5 mg/ml BSA. Prothrombin activation was started by the addition of prothrombin (preincubated at 37 °C in the same buffer). Rates of prothrombin activation were determined with the chromogenic substrate S2238 (8). The apparent K_d for dissociation of the membrane-bound factor Xa-Va complex ($K_{1/2Va}$) and the prothrombin-converting activity of this complex at [Factor Va]→∞ ($V_{Va\to\infty}$) were obtained from plots of the rate of prothrombin activation as a function of the factor Va or Xa concentration that were fitted to the equation for a single-site binding isotherm (hyperbola) via nonlinear least squares analysis.

The kinetic parameters (K_m and V_{max}) for factor Xa-catalyzed prothrombin activation were determined by measuring the rate of thrombin formation at varying prothrombin concentrations in the presence of a fixed phospholipid concentration, a limiting amount of factor Xa, and a saturating concentration factor Va or Va_{NO} as described in the previous paragraph. The kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation using nonlinear least squares analysis.

Binding Studies—Binding parameters for factor Va-membrane association were determined on planar phospholipid bilayers. The free factor Va concentrations were determined with the functional assay described above, and the concentrations of membrane-bound factor Va were determined by ellipsometry (24). Amounts of phospholipid-bound factor Va were plotted as a function of the free factor Va concentration, and the binding parameters (K_d and number of membrane binding sites) were obtained by fitting the data to the equation for a single-site binding isotherm using nonlinear least squares analysis.

RESULTS

Partial Inactivation of Factor Va by Protease Purified from Venom of N. naja oxiana—We recently purified an enzyme from the venom of *N. naja oxiana* that converts thrombin-activated factor V (factor Va) into a molecule with reduced cofactor activity (11). The effects of the venom protease on the structure and function of human factor Va were monitored with a functional assay (Fig. 1*A*) and with SDS-polyacrylamide gel electrophoresis analysis (Fig. 1*B*). Factor Va activity was determined via its ability to act as a cofactor in factor Xa-catalyzed prothrombin activation. To optimize the detection of functional differences between factor Va and factor Va_{NO} the activity assay was performed at suboptimal factor Xa, prothrombin, and phospholipid concentrations (Fig. 1; also see "Experimental Procedures"). Incubation of factor Va with venom protease resulted in a rapid decrease of cofactor function until the activity reached a plateau at approximately 15% of the activity of native factor Va (Fig. 1*A*). SDS-polyacrylamide gel electrophoresis analysis of factor Va before (Fig. 1*B*, *left lane*) and after incubation with venom protease (Fig. 1*B*, *right lane*) shows that the heavy chain of factor Va_{NO} had a slightly increased electrophoretic mobility, indicating the loss of a small peptide (M_r ~ 4000) from the heavy chain.

Amino-terminal Sequencing of Factor Va and Factor Va_{NO}—To gain more insight into the localization of the cleavage site in the factor Va molecule we subjected factor Va to amino-terminal sequencing before and after complete reaction with *N. naja oxiana* venom protease (Table I). The sequence data obtained for native factor Va indicated the presence of two polypeptide chains with amino-terminal sequences that are in agreement with the amino-terminal sequence reported for the heavy and light chain of factor Va (3).

After complete reaction of factor Va with venom protease a third amino terminus (present in about equal amounts) could be deduced from the sequence data. This new amino terminus does not originate from the venom enzyme since the amount of venom protein present in the reaction mixture was too low to be detectable in the sequence analysis (data not shown). From the fact that only one new amino-terminal sequence was formed we conclude that a single peptide bond is cleaved in factor Va during incubation with the venom protease. The newly formed amino-terminal sequence yields a unique match with the pub-

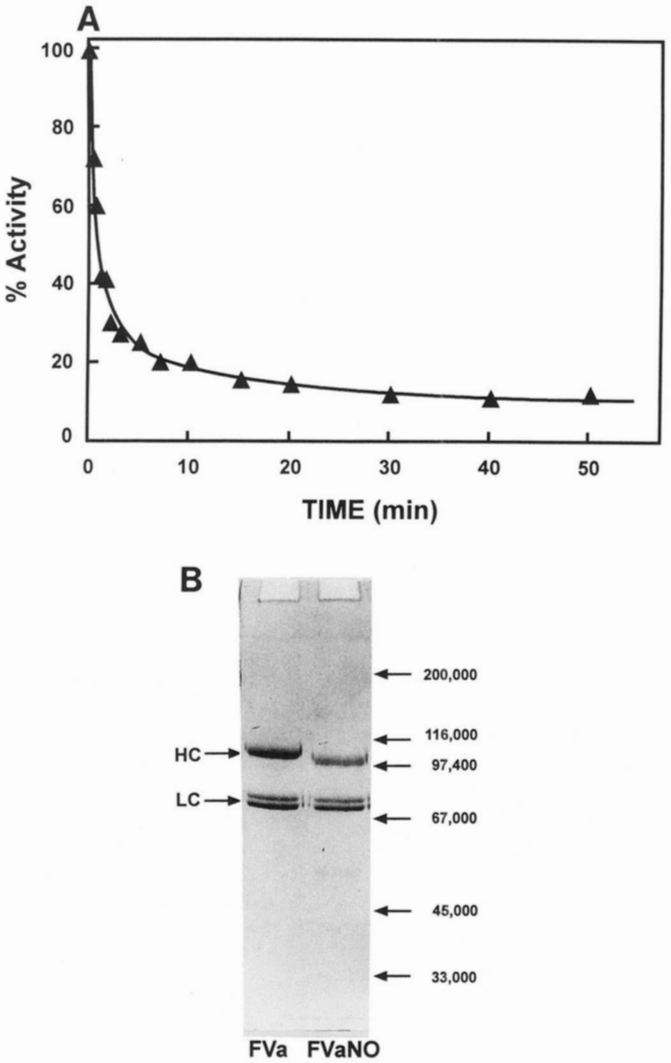

FIG. 1. **Effect of the *N. naja oxiana* venom protease on human factor Va.** Purified human factor Va (200 μg/ml) was incubated with purified *N. naja oxiana* venom protease (2 μg/ml) in 250 μl of 25 mM Hepes pH 7.5, 175 mM NaCl, and 5 mM $CaCl_2$ at 37 °C. *A*, at the indicated time points factor Va activity was determined in a reaction mixture (37 °C) containing 25 mM Hepes pH 7.5, 175 mM NaCl, 2 mM $CaCl_2$, 5 mg/ml bovine serum albumin, 0.5 nM factor Xa, 0.25 μM prothrombin, 5 μM brain phosphatidylserine/egg yolk phosphatidylcholine (5:95, M/M). Further details of the assay procedure are described under "Experimental Procedures." *B*, before addition of venom protease (*lane FVa*) and after complete reaction (50 min, *lane* FVa_{NO}) 25-μl aliquots were taken for gel electrophoretic analysis on a 7.5% polyacrylamide slab gel (5% stacking gel) in the presence of sodium dodecyl sulfate and 5% 2-mercaptoethanol. The positions of the Va heavy chain (*HC*) and the light chain doublet (*LC*) are indicated together with the positions of molecular size markers run on a separate *lane* on the gel.

lished sequence of factor V at Asp^{683}–Glu^{690} (3), a region located in the heavy chain domain of factor Va.

HPLC of Factor V and Factor Va_{NO}—Factor Va and factor Va_{NO} preparations were subjected to reversed-phase chromatography. In the case of factor Va a broad protein peak that elutes between 33 and 45 min presumably contains the heavy and light chains of factor Va (Fig. 2*A*). A similar broad protein peak was observed in the factor Va_{NO} preparation (Fig. 2*B*). However, the chromatogram of factor Va_{NO} showed one major additional peptide peak eluting at 24.6 min. This peptide peak, which was neither present in factor Va (Fig. 2*A*) nor in the purified venom protease preparation (data not shown), was collected for sequence analysis. The amino acid sequence of this peptide (Table II) appeared to be identical to the last 27 amino acids from the carboxyl-terminal part of the heavy chain of factor Va (3). The small peaks eluting between 28 and 32 min did not contain material that could be sequenced.

From these data we conclude that incubation of factor Va with the purified protease from *N. naja oxiana* venom resulted in cleavage of the peptide bond between His^{682} and Asp^{683}. Because of this cleavage a peptide of 27 amino acids (with a calculated M_r of about 3140) is removed. This is in reasonable agreement with the change in apparent M_r of the factor Va heavy chain observed in the SDS gels after reaction of factor Va with venom protease (*cf.* Fig. 1*B*).

Functional Properties of Factor Va_{NO}—Optimal cofactor activity of factor Va in prothrombin activation requires interaction of factor Va with procoagulant membranes, factor Xa, and prothrombin. To examine which of these functions was impaired in *N. naja oxiana*-treated factor Va we compared factor Va and factor Va_{NO} in direct binding studies and in a kinetic analysis of prothrombin activation.

Fig. 3 shows an experiment in which we tested the ability of factors Va and Va_{NO} to bind to a planar lipid bilayer composed of 20 mol % DOPS in DOPC. Factors Va and Va_{NO} bound equally well to the membrane, and half-maximal binding was observed at 2.2 nM factor Va and 1.7 nM factor Va_{NO}. At saturating concentrations of factors Va and Va_{NO} the amounts of bound protein were 0.34 μg of factor Va/cm^2 of phospholipid and 0.35 μg of factor Va_{NO}/cm^2 of phospholipid, respectively. Considering that 1 cm^2 of a phospholipid bilayer contains 0.4 μg of phospholipid and that the molecular weights of factor Va and the phospholipid used are ~180,000 and ~800, respectively, it can be calculated that the phospholipid:factor Va ratio (mol/mol) at saturation is 265 for factor Va and 257 for factor Va_{NO}. Thus, it appears that the cleavage in the factor Va heavy chain by *N. naja oxiana* did not result in a change in lipid binding properties of the factor Va molecule. These data support the concept that it is the light chain region of factor Va that is actually responsible for lipid binding (25–28).

To compare the ability of factors Va and Va_{NO} to assemble into a membrane-bound prothrombinase complex we determined initial steady-state rates of prothrombin activation at a limited amount of factor Xa as a function of the factor Va or factor Va_{NO} concentration (Fig. 4*A*). Low amounts of factor Va were required for full expression of prothrombinase activity, whereas much higher amounts of Va_{NO} were needed in order to obtain similar activation rates. Half-maximal prothrombinase complex formation was observed at 0.25 and 2.01 nM factor Va and factor Va_{NO}, respectively. The double-reciprocal plot shown in Fig. 4*B* shows that the intercepts at the *y*-axis were approximately the same for factors Va and Va_{NO}. Thus, once formed the factor Xa-Va and factor Xa-Va_{NO} complexes are equally capable of activating prothrombin.

With respect to the different cofactor activities of factor Va and Va_{NO}, the possibility had to be ruled out that factor Va_{NO} lacks functional activity and that its cofactor activity is due to a small (~15%) remaining amount of factor Va. This possibility is excluded by the observations that 1) titrations of fixed limiting amounts of factor Va or Va_{NO} with factor Xa yield a $K_{1/2Xa\text{-}Va}$ of 0.24 and 2.27 nM for factor Xa-Va and Xa-Va_{NO} complexes, respectively (Table III) and 2) a functional factor Va assay performed at saturating factor Xa (5 nM), prothrombin (3 μM), and phospholipid (50 μM DOPS/DOPC, 20:80, M/M) yields identical activities for factor Va and factor Va_{NO} (data not shown).

We also determined the kinetic parameters of prothrombin activation at a fixed factor Xa and phospholipid concentration and at saturating concentrations of factor Va or Va_{NO}. Fig. 5*A* shows the rates of thrombin formation obtained for the factor Xa-Va and factor Xa-Va_{NO} complexes as a function of prothrom-

TABLE I

Amino-terminal sequences of factor Va before and after incubation with the venom protease from N. naja oxiana

Human factor Va (30 μg/ml) was incubated for 1 h at 37 °C in 25 mM Hepes pH 7.5, 175 mM NaCl, 2 mM $CaCl_2$ either in the absence or presence of *N. naja oxiana* venom protease (0.3 μg/ml). Both factor Va samples were subjected to amino-terminal sequencing as described under "Experimental Procedures."

Cycle no.	Factor Va			Factor Va + *N. naja oxiana*		
	Residue	Factor V residues 1–8[a]	Factor V residues 1546–1553[a]	Residue	New NH_2 terminus	Factor V residues 683–690[a]
	pmol			*pmol*		
1	A (31.1) S (14.4) D (22.9)[b]	A	S	A (30.5) S (15.4) D (68.0)	(D)	D
2	Q (29.6) N (15.6)	Q	N	Q (39.6) N (18.7) R (10.6)	R	R
3	L (17.4) N (20.3)	L	N	L (22.7) N (19.7)	L	L
4	R (19.1) G (16.6)	R	G	R (22.6) G (16.5) E (13.4)	E	E
5	Q (16.1) N (19.1)	Q	N	Q (19.7) N (17.5) P (15.5)	P	P
6	F (13.4)	F	R	F (14.6) E (19.0)	E	E
7	Y (14.0)	Y	R	Y (12.5)	?	D
8	V (14.0)	V	N	V (16.0) E (19.8)	E	E

[a] Amino-terminal sequence of the heavy chain (residues 1–8) and light chain (residues 1546–1553) of factor Va (from Ref. 3).
[b] Part of the aspartic acid in the first cycle is most likely accounted for by coelution of phenylisothiocyanate-NH_4.

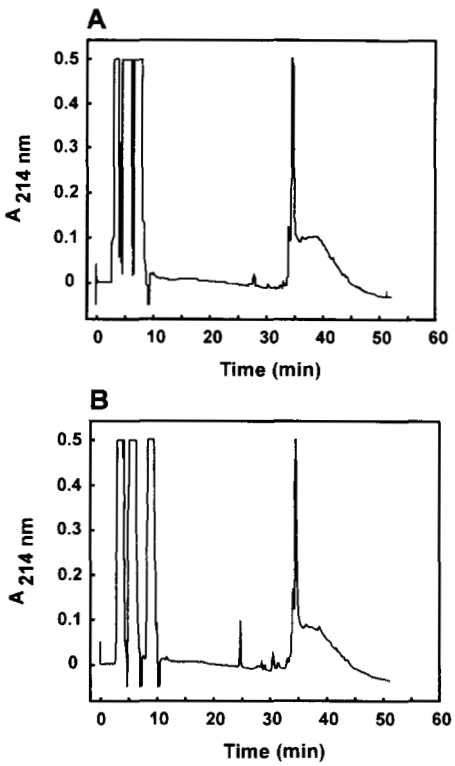

FIG. 2. **Reversed-phase chromatography of factor Va and factor Va_{NO}.** Factor Va (250 nM) was incubated for 10 min in 25 mM Hepes pH 7.5, 175 mM NaCl, and 5 mM $CaCl_2$ at 37 °C either in the absence (*A*) or presence (*B*) of 1 μg/ml purified venom protease from *N. naja oxiana*. 600 μl of these mixtures were applied to an Aquapore C18 column, concentrated for 10 min with 0.1% trifluoroacetic acid in 12% acetonitrile and eluted (40 min) with a linear gradient of acetonitrile (12–48%) in 0.1% trifluoroacetic acid. Further details are given under "Experimental Procedures."

TABLE II

Amino acid sequence of peptide isolated from factor Va_{NO} preparations

The peptide isolated from factor Va_{NO} preparations (Fig. 2*B*) was subjected to amino acid sequencing as described under "Experimental Procedures." The amino acid sequence indicated was determined by automated call using the software supplied with the sequencer. The number in parentheses indicates the background-corrected picomoles at a given cycle. It is most likely that the first amino acid is Asp (9.6 pmol). It could not, however, be positively identified because of impurities in the first cycle. The last amino acid (Arg) showed a low yield caused by a strong washout of the last residue.

Cycle no.	Amino acid	Cycle no.	Amino acid
	pmol		*pmol*
1	?	14	Y (5.7)
2	R (6.4)	15	D (7.4)
3	L (24.7)	16	Y (8.0)
4	E (17.8)	17	Q (4.5)
5	P (12.8)	18	N (3.4)
6	E (11.6)	19	R (2.9)
7	D (7.4)	20	L (2.2)
8	E (9.8)	21	A (4.7)
9	E (11.3)	22	A (7.7)
10	S (1.3)	23	A (10.7)
11	D (5.2)	24	L (2.1)
12	A (9.2)	25	G (3.2)
13	D (7.2)	26	I (2.7)
		27	R (0.2)

bin concentration. From the Lineweaver-Burk plots shown in Fig. 5*B* it can be concluded that the reaction adhered to overall Michaelis-Menten kinetics. With the factor Xa-Va complex the reaction was characterized by a K_m for prothrombin of 0.24 μM and a V_{max} of 6860 mol of prothrombin activated per min per mol of factor Xa. This is in reasonable agreement with data reported earlier (23, 28). In the case of the factor Xa-Va_{NO} complex the K_m was less favorable (0.83 μM), whereas V_{max} was slightly higher (7685 mol of prothrombin activated per min per mol of factor Xa).

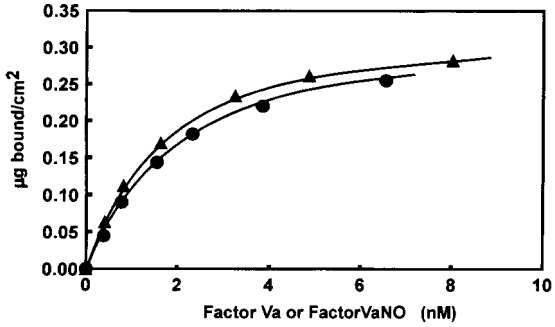

FIG. 3. **Binding of factor Va and Va_{NO} to planar phospholipid bilayers.** Binding of factor Va (●) and factor Va_{NO} (▲) to planar phospholipid bilayers (DOPS/DOPC, 20:80, M/M) was determined by ellipsometry (24) in a buffer containing 25 mM Hepes pH 7.5 at 37 °C, 175 mM NaCl, 2 mM $CaCl_2$, and 5 mg/ml BSA. The *solid lines* represent *hyperbolas* obtained by a nonlinear least squares fit of the data to a single-site binding isotherm. The binding parameters describing these *hyperbolas* are K_d(Va) = 2.2 nM and 0.34 μg of factor Va bound per cm^2 of phospholipid at saturating factor Va or $K_d(Va_{NO})$ = 1.7 nM and 0.35 μg of factor Va_{NO} bound per cm^2 of phospholipid at saturating factor Va_{NO}.

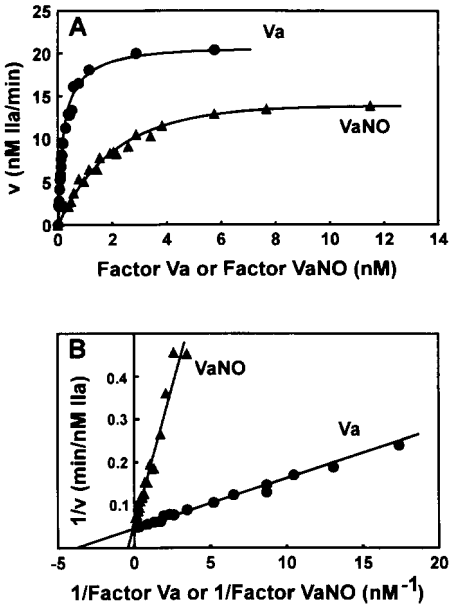

FIG. 4. **Comparison of cofactor activities of factor Va and Va_{NO}.** Initial rates of prothrombin activation were determined at 37 °C at a fixed, limiting concentration of factor Xa (3 pM) in 250 μl of buffer containing 25 mM Hepes pH 7.5, 175 mM NaCl, 2 mM $CaCl_2$, 5 mg/ml BSA, 25 μM phospholipid vesicles (DOPS/DOPC, 5:95, M/M), 1 μM prothrombin, and varying amounts of factor Va (●) or Va_{NO} (▲) as described under "Experimental Procedures." *A*, rate of prothrombin activation as a function of factor Va or Va_{NO} concentration. The *solid lines* represent *hyperbolas* obtained after fitting the data with $K_{1/2Va}$ = 0.25 nM and $V_{Va\rightarrow\infty}$ = 21.7 nM IIa/min (●) and with $K_{1/2Va_{NO}}$ = 2.01 nM and $V_{Va_{NO}\rightarrow\infty}$ = 16.9 nM IIa/min (▲). *B*, double-reciprocal plot of the same data.

Dissociation of Peptide from Factor Va_{NO}—It may be argued that the peptides resulting from cleavage of factor Va by the venom protease remain associated with the factor Va_{NO} molecule through noncovalent interactions. Unfortunately, no methods are available to detect the possible separation of the peptide from factor Va_{NO} at the low factor Va_{NO} concentrations employed in the kinetic experiments. We were able to show, however, that inactivation of high concentrations of factor Va (250 nM) with the venom protease and subsequent gel filtration on a small desalting column (9.1-ml Sephadex G-25M) resulted in removal of the majority (~70%) of the peptide from the factor Va_{NO} fraction. This indicates that the peptide is in a normal association-dissociation equilibrium with factor Va_{NO} at high concentrations, and it is likely that it will be fully released from factor Va_{NO} at the low concentrations employed in the kinetic experiments.

TABLE III
Comparison of functional properties of factors Va and Va_{NO}

This table summarizes the different properties of factor Va and Va_{NO} obtained from the data presented in Figs. 3–5. For details see text and the legends to the figures.

	Factor Va	Factor Va_{NO}
K_dPL[a]	2.7 nM	1.7 nM
n[b]	265	257
$K_{1/2Xa\text{-}Va}$[c]	0.25 nM	2.01 nM
$K_{1/2Xa\text{-}Va}$[d]	0.24 nM	2.27 nM
K_m[e]	0.24 μM	0.83 μM
k_{cat}[e]	114 s^{-1}	128 s^{-1}

[a] Dissociation constant for membrane factor Va or membrane factor Va_{NO} complex (*cf.* Fig. 3).
[b] Phospholipid:factor Va or Va_{NO} ratio (mol/mol) at saturation with factor Va or Va_{NO} (*cf.* Fig. 3).
[c] Apparent dissociation constant for the membrane-bound factor Xa-Va or Xa-Va_{NO} complex determined at limiting factor Xa concentrations (*cf.* Fig. 4).
[d] Apparent dissociation constant for the membrane-bound factor Xa-Va or Xa-Va_{NO} complex determined at limiting factor Va/Va_{NO} concentrations (data not shown).
[e] K_m and k_{cat}, kinetic parameters of prothrombin activation by the factor Xa-Va or factor Xa-Va_{NO} complex (*cf.* Fig. 5).

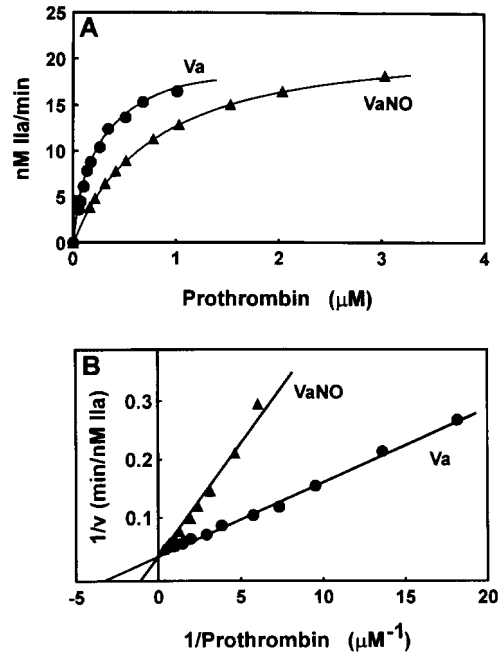

FIG. 5. **Prothrombin activation by Xa-Va and Xa-Va_{NO} as a function of prothrombin concentration.** Initial rates of prothrombin activation were determined as described under "Experimental Procedures" at a fixed, limiting concentration of factor Xa (3 pM) in a reaction mixture (37 °C) containing 25 mM Hepes pH 7.5, 175 mM NaCl, 2 mM $CaCl_2$, 5 mg/ml BSA, 25 μM phospholipid vesicles (DOPS/DOPC, 5:95, M/M), saturating amounts of Va (5 nM, ●) or Va_{NO} (35 nM, ▲), and varying amounts of prothrombin. *A*, initial rate of activation as a function of prothrombin concentration. The *solid lines* represent *hyperbolas* with K_m = 0.24 μM and V_{max} = 6860 mol of IIa/min/mol of factor Xa-Va (●) and with K_m = 0.83 and V_{max} = 7685 mol of IIa/min/mol of factor Xa-Va_{NO} (▲) that were obtained by fitting the data to the Michaelis-Menten equation using nonlinear least squares analysis. *B*, Lineweaver-Burk plots of the same data.

DISCUSSION

Factor Va exerts multiple effects in factor Xa-catalyzed prothrombin activation. The cofactor strongly increases the cata-

lytic turnover (k_{cat}) of the enzyme factor Xa and promotes the assembly of productive enzyme-substrate complexes at the procoagulant membrane by stimulating the binding of factor Xa and prothrombin to the membrane. Thus, it appears that factor Va is a multifunctional protein that promotes prothrombin activation via interaction with phospholipids, factor Xa, and prothrombin. The phospholipid binding site of factor Va appears to be located on the light chain (25–28), and prothrombin has been reported to interact with the heavy chain (17), whereas both the heavy and light chains of factor Va have been implicated to interact with factor Xa (17, 29, 30).

The data presented in this paper give additional insight into the domains of the Va molecule that are involved in the interaction with factor Xa and prothrombin. Using a protease purified from the venom of *N. naja oxiana* we obtained a factor Va molecule (factor Va_{NO}) that lacks 27 amino acid residues from the carboxyl terminus of the heavy chain and that is further characterized by a greatly reduced cofactor activity.

Table III summarizes the functional properties of factor Va and factor Va_{NO}. Both factor Va and factor Va_{NO} bind equally well to negatively charged phospholipids. This is in agreement with earlier reports that indicate that the heavy chain of factor Va is not involved in membrane binding. The apparent K_d for Xa-Va complex formation at the phospholipid membrane is, however, about 1 order of magnitude less favorable in the truncated factor Va_{NO} molecule (Table III and Fig. 4). This strongly suggests that the carboxyl-terminal residues of the heavy chain are important for the interaction of factor Va with factor Xa. Once formed, the factor Xa-Va_{NO} complex also has somewhat less favorable kinetic parameters for prothrombin activation as evidenced by the increase in K_m for prothrombin (Table III and Fig. 5). The ability of factor Va to increase the enzymatic turnover (k_{cat}) of prothrombin activation is not affected in the factor Va_{NO} molecule (Table III). Thus, the final 27 carboxyl-terminal residues of the factor Va heavy chain do not play a role in the increase of the k_{cat} of prothrombin activation, but these residues are apparently involved in complex formation of factor Va with both factor Xa and prothrombin.

The fact that the carboxyl terminus of the factor Va heavy chain plays a role in protein-protein interactions between factor Va and other proteins can be appreciated on the basis of the primary structure of factor Va. The carboxyl terminus of the heavy chain of factor Va is highly acidic (3) and can, therefore, be expected to be at the surface of the molecule and to be readily available for interaction with other molecules. In this respect it is interesting to note that Kalafatis *et al.* (31) recently reported that the heavy chain of bovine factor Va can be phosphorylated at Ser^{690} and that this phosphorylation renders the molecule more susceptible to inactivation by activated protein C. No data were given, however, on the effect of phosphorylation on the interaction of factor Va with factor Xa or prothrombin. Whether such phosphorylation can also occur in the human molecule is as yet also unknown. The literature data (3, 31) emphasize, however, that this region of the factor Va heavy chain is accessible for interaction with other macromolecules and plays an important role in the expression and regulation of factor Va cofactor activity.

The cleavage site at which the protease purified from *N. naja oxiana* venom cleaves factor Va (His^{682}-Asp^{683}) is an unusual target site for proteolytic enzymes. There is, however, a report on an abnormal fibrinogen (in which Arg^{16} of the Aα-chain is replaced by His^{16}) that is cleaved by thrombin after this histidine residue (32). It is also possible that the His^{682}-Asp^{683} bond is cleaved by an endoproteinase Asp-N (33) with specificity toward the particular amino acid sequence that is cleaved in the heavy chain of factor Va. It is interesting, therefore, to further study this snake venom protease in order to gain more insight into the molecular basis for its proteolytic specificity.

Acknowledgments—We thank Rita Janssen and Dr. George Willems for performing the binding experiments on the ellipsometer.

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